



Protection against H7N3 high pathogenicity avian influenza in chickens immunized with a recombinant fowlpox and an inactivated avian influenza vaccines

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ABSTRACT

Beginning on June 2012, an H7N3 highly pathogenic avian influenza (HPAI) epizootic was reported in the State of Jalisco (Mexico), with some 22.4 million chickens that died, were slaughtered on affected farms or were preemptively culled on neighboring farms. In the current study, layer chickens were vaccinated with a recombinant fowlpox virus vaccine containing a low pathogenic AI (LPAI) H7 gene insert (rFPV-H7-AIV) and an inactivated oil-emulsified H7N3 AIV vaccine, and subsequently challenged against the Jalisco H7N3 HPAIV. All vaccine combinations provided similar and significant protection against mortality, morbidity, and shedding of challenge virus from the respiratory and gastrointestinal tracts. Serological data also suggested analogous protection from HPAIV among immunized birds. Control of the recent Jalisco AIV infection could be achieved by using various combinations of the two vaccines tested. Even though a single dose of rFPV-H7-AIV vaccine at 1-day-of-age would be the most pragmatic option, optimal protection may require a second dose of vaccine administered in the field.

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1. Introduction

During the past decade, avian influenza (AI), and particularly H5N1 high pathogenicity AI (HPAI), has become one of the major zoonotic health issues facing commercial poultry, wildlife, and humans in Asia, Africa, and parts of Eastern Europe [1]. Diverse HPAI viruses (HPAIV) have been involved in other numerous outbreaks in poultry and wild birds worldwide, with a dramatic economic and social impact especially due to the high number of birds that have died and large number of poultry culled in stamping-out programs [2].

Beginning on June 2012, an H7N3 HPAI epizootic was reported in the State of Jalisco (Mexico), an area with high poultry density. In total, 44 commercial layer farms were affected, with some 22.4 million chickens dead, were culled on affected farms or were preemptively culled on neighboring farms. Biosecurity strategies consisting in quarantine, depopulation, movement control, and surveillance were established. On 26 July 2012, an immunization campaign using an H7N3 inactivated vaccine was implemented in layer chickens within the control zone in conjunction with the other control measures [3,4]. Besides the economic consequences of such outbreaks, it is worth mentioning that influenza A (H7) infection in

humans associated with exposure to infected poultry was reported during the H7N3 HPAI epizootics in Jalisco [5].

The use of vaccines is a justifiable tool for control of HPAI when implemented properly and in combination with strict epidemiological surveillance and biosecurity measures [6]. Indeed, immunization has been a more cost-effective and feasible tool than stamping-out programs alone in some developing countries [7–9]. Vaccination was first implemented against HPAI in Mexico during 1995, when an inactivated oil-emulsified vaccine, made from a 1994 precursor H5N2 low pathogenicity AIV (LPAIV), was used [8]. Immunization of the birds was one tool of the control program which led to successfully eradicate the H5N2 HPAIV, but its LPAIV precursor has continued to circulate in central Mexico. As a result, vaccines against notifiable LPAI (LPNAI, i.e. H5 and H7 subtypes) have been continuously and extensively used since then, making Mexico the top vaccine user in the world for LPNAI [9]. Currently, a typical immunization program for H5N2 LPNAI control in Mexico may consist of an initial dose at 1- to 4-days-of-age (d) of a virus-vectorized vaccine with an H5 hemagglutinin (HA) gene insert in a fowlpox virus [rFPV-H5-AIV], Newcastle disease virus or turkey herpesvirus, followed by a prime-boost application of an inactivated AIV vaccine at 10 to 14d. Such use of inactivated vaccines requires handling and injection of individual chickens on the farm, creating a compromised biosecurity situation with vaccinators coming and going between multiple farms for the twice vaccination. A single dose of rFPV vaccine would have the advantage of being administered

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in the hatchery, before the chicks are sent to the farm, and could be a primary protective vaccine or used in a prime-boost protocol to optimize protection in the field with only one vaccination on the farm.

Matching the HA subtype of the current Mexican epizootic, the present study assessed the protective efficacy of two different H7 AIV vaccines, an rFPV-H7-AIV vaccine and an inactivated H7N3 AIV vaccine, in different combinations with the aim to establish whether single-vaccinated layer chickens would be protected against the 2012 Mexican H7N3 HPAIV or if a second immunization in the field would be required.

2. Materials and methods

2.1. Animals

Specific-pathogen-free (SPF) White Leghorn (WL) chicks from Southeast Poultry Research Laboratory (SEPRL) in house flocks were used. Each group of birds was housed separately in negative pressured isolators with HEPA-filtered air within the animal biosafety level 2 (ABSL-2) facilities of SEPRL during vaccination trials, and they were subsequently transferred to ABSL-3's isolators for challenge. Feed and water were provided *ad libitum* throughout the experiment. All procedures were performed according to the requirements of the protocol approved by the Institutional Laboratory Animal Care and Use Committee.

2.2. Viruses

A Mexican HPAIV, isolated from the index case of the 2012 H7N3 HPAI epizootic, A/Chicken/Jalisco/12283/12 (H7N3) (Jalisco/12, GenBank accession number JX908509.1) was used as the challenge virus [3]. The virus was propagated and titrated by allantoic sac inoculation of 9d embryonated chicken eggs (ECE) by standard methods [10]. Two different vaccines were tested: 1) an rFPV vaccine containing an H7 LPAIV gene insert from the North American strain A/Turkey/Virginia/66/02 (H7N2, GenBank accession number AY240913) (rFPV-2155, Merial-Select Inc., Gainesville, GA) (rFPV-H7-AIV); and 2) an experimental inactivated H7 AIV vaccine based on official Mexican vaccine seed strain A/Cinnamon Teal/Mexico/2817/2006 H7N3 (H7N3)(CT/Mex/06, GenBank accession number KC669389). The inactivated vaccine was prepared with an oil-emulsified Montanide ISA70V adjuvant (SEPPIC, Inc., Paris, France) using infectious allantoic fluid previously inactivated with 0.1% beta-propiolactone. The vaccines were subcutaneously administered in the nape of the head in a dose of $10^{3.5}$ mean tissue culture infectious doses (TCID₅₀) of rFPV-H7-AIV (in 0.2 ml) and 512 HA units of CT/Mex/06 (in 0.5 ml).

2.3. Experimental design

Fifty WL chickens were randomly distributed into five groups of ten birds each. Groups 2 and 3 were vaccinated with the rFPV-H7-AIV vaccine at 1d, group 3 receiving a boost CT/Mex/06 vaccine at 3wks. Groups 4 and 5 were vaccinated with the CT/Mex/06 vaccine at 1d, group 5 receiving a boost CT/Mex/06 vaccine at 3wks. The sham vaccination group (group 1) was inoculated with the rFPV-H7-AIV vaccines' diluent at 1d and the oil emulsion adjuvant at 3wks. At 3-weeks post-second vaccination (6-weeks-of-age), all birds were bled to evaluate antibody titers, and they were challenged by the intranasal route with 10^6 EID₅₀ of Jalisco/12 HPAIV in a volume of 0.1 ml. The inoculum titer was subsequently verified as $10^{6.1}$ mean chicken embryo infectious doses (EID₅₀)/0.1 ml by back titration in ECE.

2.4. Sampling

Chickens were monitored daily for clinical signs and mortality. Oropharyngeal (OS) and cloacal (CS) swabs were collected at 2 and 4 days post-challenge (dpc), placed in brain heart infusion (BHI) medium with 2X antibiotics, and stored at -70°C until tested. In order to address how vaccination could influence on the virus pathogenesis, progress and extent of infection, severity of lesions, and presence of the AIV in tissues, up to two dead birds per group were submitted to necropsy to evaluate gross lesions and obtain samples for histopathological studies. The following tissues were collected: duodenum, jejunum, cecal tonsil, pancreas, liver, kidney, adrenal gland, nasal turbinates, trachea, lung, heart, breast muscle, bone marrow, spleen, cloacal bursa, thymus, and brain. At the end of the experiment (14 dpc, 8-weeks-of-age), surviving birds were bled to evaluate antibody titers and euthanized by intramuscular anesthesia with ketamine (10 g/kg body weight, Imalgene® 1000, Merial, Lyon, France) followed by cervical dislocation.

2.5. Histopathology and immunohistochemistry

Necropsies and tissue sampling were performed according to a standard protocol [11]. Briefly, after fixation in 10% neutral buffered formalin, tissue sections were embedded in paraffin. In addition, the nasal cavity was decalcified for 2 days. Sections were made at 5 μm and were routinely processed for hematoxylin/eosin (HE) staining. A duplicate section was stained for immunohistochemistry (IHC) by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A 1:2000 dilution of a mouse-derived monoclonal antibody (P13C11, developed at SEPRL) specific for type A influenza virus nucleoprotein was applied and allowed to incubate overnight at 4°C . As a secondary antibody, a biotinylated goat anti-mouse IgG antibody using a biotin-streptavidin detection system (Supersensitive Multilink Immunodetection System, Biogenex) was applied. Fast Red TR (Biogenex) served as the substrate chromagen, and hematoxylin was used as a counterstain. All tissues were systematically screened for microscopic lesions and viral antigen staining.

2.6. Viral RNA quantification in oropharyngeal and cloacal swabs

Swabs were processed for quantitative real-time RT-PCR (qRT-PCR) to determine viral RNA titers. Viral RNA from OS and CS was extracted using MagMAX™-96 AI/ND Viral RNA Isolation Kit® (Ambion, Inc.) following the manufacturer's instructions. The resulting viral RNA extracts were quantified by one-step qRT-PCR which targets the influenza matrix gene [12] using 7500 FAST Real-time PCR System (Applied Biosystems, Foster City, CA, USA) and the AgPath-ID OneStep RT-PCR kit (Ambion, Inc.). The standard curve for viral RNA quantification was established with RNA extracted from dilutions of the same titrated stock of the challenge virus, and it was run in each plate. The limit of detection was determined to be $10^{3.5}$ EID₅₀/ml; therefore qRT-PCR negative samples were treated as $\leq 10^{3.4}$ EID₅₀/ml.

2.7. Serology

Hemagglutinin inhibition (HI) assays were carried out to evaluate antibody levels in vaccinated birds both pre- and post-challenge. The assay antigens were specific for the vaccine seed viruses and the challenge virus, therefore: A/Turkey/Virginia/66/02 (H7N2), A/Cinnamon teal/Mexico/2817/06 (H7N3), and A/Chicken/Jalisco/12283/12 (H7N3). The antigens were prepared as previously described [13], and the HI assays were performed according to standard procedures [14]. Titers were expressed as

Table 1

Morbidity, mortality, and mean viral shedding data from WL chickens vaccinated with either an rFPV-H7-AIV vaccine or an inactivated AIV vaccine at different time points and subsequently challenged with 10^6 EID₅₀ Jalisco/12 H7N3 HPAIV.

Group	Vaccine virus (age of vaccination)	Morbidity	Mortality (MDT*)	Viral RNA detection† (log ₁₀ EID ₅₀ titer/ml)			
				2 dpc		4 dpc	
				OS	CS	OS	CS
1	Sham (1d + 3wks)	8/10 ^a	8/10 ^a (2.25)	8/10 ^a ($\leq 5.17^A$)	8/10 ^a ($\leq 5.28^A$)	0/2 [§] ($\leq 3.4^§$)	0/2 [§] ($\leq 3.4^§$)
2	rFPV-H7-AIV (1d)	0/10 ^b	0/10 ^b	0/10 ^b ($\leq 3.4^B$)	0/10 ^b ($\leq 3.4^B$)	1/10 ^b ($\leq 3.45^B$)	1/10 ^b ($\leq 3.64^B$)
3	rFPV-H7-AIV (1d) + CT/Mex/06 (3wks)	0/10 ^b	0/10 ^b	0/10 ^b ($\leq 3.4^B$)	0/10 ^b ($\leq 3.4^B$)	0/10 ^b ($\leq 3.4^B$)	0/10 ^b ($\leq 3.4^B$)
4	CT/Mex/06 (1d)	1/10 ^b	1/10 ^b (4.00)	2/10 ^b ($\leq 3.61^B$)	1/10 ^b ($\leq 3.53^B$)	3/10 ^b ($\leq 3.87^B$)	1/10 ^b ($\leq 3.64^B$)
5	CT/Mex/06 (1d) + CT/Mex/06 (3wks)	0/10 ^b	0/10 ^b	0/10 ^b ($\leq 3.4^B$)	0/10 ^b ($\leq 3.4^B$)	0/10 ^b ($\leq 3.4^B$)	0/10 ^b ($\leq 3.4^B$)

MDT, mean death times; qRT-PCR, quantitative real time PCR; OS, oropharyngeal swabs; CS, cloacal swabs; dpc, days post challenge; rFPV-H7-AIV, rFPV-2155 vaccine; CT/Mex/06, inactivated A/Cinnamon Teal/Mexico/2817/2006 vaccine; d, day-of-age; wks, weeks-of-age.; *#dead birds x dpc/total dead birds (expressed as dpc); †#positive at qRT-PCR/total; §The number of positive birds was too low for statistical purposes.; For statistical purposes, all OS and CS without viral RNA detection were given a numeric value of $10^{3.4}$ EID₅₀/ml, which represents the lowest detectable level of viral RNA with the qRT-PCR used.; Different superscript lowercase letters denote significant difference for mortality and for number of positive birds on qRT-PCR between groups; Fisher's exact test, $P < 0.05$; Different superscript uppercase letters denote significant difference for mean viral titers between groups; Mann-Whitney test, $P < 0.05$.

geometric mean titers (GMT-log₂). Samples with titers below 3 log₂ GMT were considered negative; samples reaching maximum detectable titers were considered to have 14 log₂ GMT.

2.8. Statistical analysis

Morbidity, mortality, and number of birds shedding virus were tested for statistical significance with Fisher's exact test. Significant difference for mean viral titers between groups was analyzed using Mann-Whitney test. Antibody levels were tested for statistical significance with Kruskal-Wallis and Mann-Whitney tests. All tests were performed using Graph Pad Prism version 5 for Windows (Graph Pad Software, La Jolla, CA, USA, www.graphpad.com). A P -value of <0.05 was considered to be significant.

3. Results

3.1. Protection against clinical signs and mortality

Chickens in the sham immunized group (group 1) developed clinical signs (80%) and died (80%) between 2 and 3 dpc (mean death time [MDT] = 2.25 dpc) (Table 1). Most of the birds (5/8) were found dead without overt clinical signs (peracute disease), but some of them (3/8) showed nonspecific clinical signs like ruffled feathers, lethargy, anorexia, and prostration a few hours before death.

Respiratory distress signs were also observed, including severe dyspnea as was with facial edema, swelling of the infraorbital sinuses, and conjunctivitis. Copious nasal discharge was also observed. In contrast, immunization with the inactivated CT/Mex/06 vaccine at 3wks, receiving at 1d either rFPV-H7-AIV (group 3) or CT/Mex/06 (group 5), conferred complete clinical protection, thus resulting in no clinical signs or death (Table 1). Only one bird from the rFPV-H7-AIV (1d) vaccinated group (group 2) experienced mild depression and ruffled feathers at 4 dpc, and subsequently recovered. One bird of the group vaccinated with CT/Mex/06 (1d) (group 4) died on 4 dpc without previous clinical signs (Table 1). Interestingly, at the same time point the other birds of group 4 exhibited anorexia.

3.2. Pathobiology in dead birds

Two sham vaccinated birds (group 1, one euthanized and one found dead) at 2 dpc, and the only found dead bird immunized with CT/Mex/06 (1d) (group 4) at 4 dpc, were submitted to necropsy. Gross lesions were similar in all the chickens examined, all of them consistent with HPAIV infection. Histopathological findings correlated well with viral antigen staining in tissues, being consistent among birds and with HPAI infection (Fig. 1). Viral antigen was present in several organs of all the examined birds, suggesting systemic infection regardless of being sham (group 1) or immunized

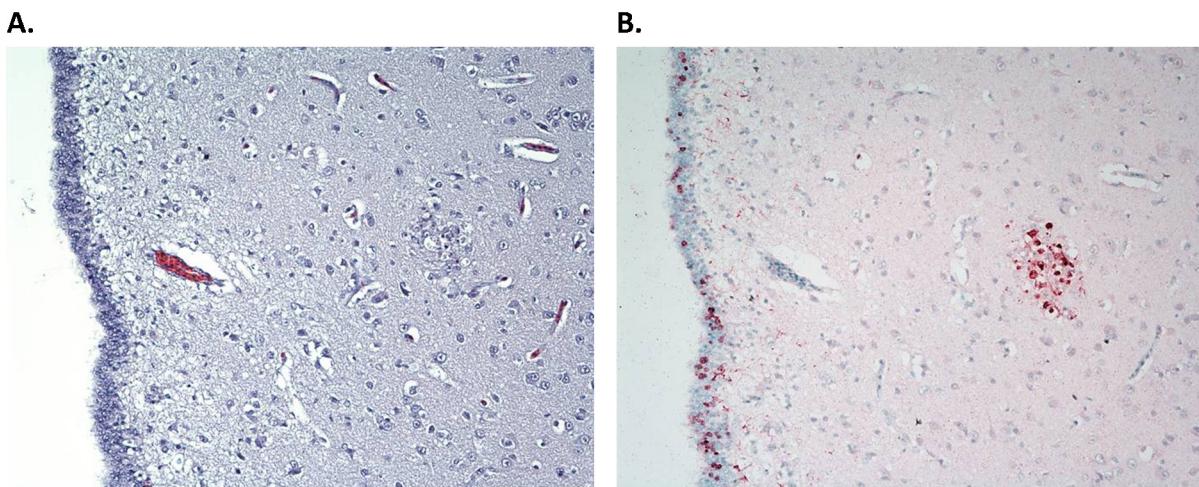


Fig. 1. Histopathology and immunohistochemical staining for AIV antigen in brain from a sham vaccinated WL chicken and subsequently challenged with 10^6 EID₅₀ Jalisco/12 H7N3 HPAIV, dead at 2 dpc. A. Encephalomalacia and necrosis of ependymal cells of the ventricles (HE, 20 \times). B. Viral antigen in neurons, glial cells, ependymal cells, and vascular endothelial cells (IHC, 20 \times).

Table 2

Serological data from WL chickens vaccinated with either an rFPV-H7-AIV vaccine or an inactivated AIV vaccine at different time points and subsequently challenged with 10^6 EID₅₀ Jalisco/12 H7N3 HPAIV.

Group	Vaccine virus (age of vaccination)	HI serology* (GMT-log ₂)					
		A/Turkey/Virginia/66/02		A/Cinnamon Teal/Mexico/2817/2006		A/Chicken/Jalisco/12283/12	
		Pre-challenge	Post-challenge	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
1	Sham (1d + 3wks) [†]	0/10	0/2	0/10	0/2	0/10	0/2
2	rFPV-H7-AIV (1d)	10/10 (7.3 ^a) ^a	10/10 (8.1 ^A) ^A	10/10 (6.0 ^b) ^b	10/10 (5.5 ^A) ^A	10/10 (4.1 ^A) ^c	8/10 (6.9 ^A) ^A
3	rFPV-H7-AIV (1d) + CT/Mex/06 (3wks)	10/10 (9.1 ^B) ^a	10/10 (9.5 ^{A,B}) ^A	10/10 (10.0 ^B) ^a	10/10 (7.7 ^B) ^B	10/10 (9.3 ^B) ^a	10/10 (8.8 ^A) ^A
4	CT/Mex/06 (1d)	9/10 (4.8 ^C) ^a	9/9 (6.9 ^{A,C}) ^A	10/10 (7.4 ^C) ^b	9/9 (6.8 ^C) ^A	9/10 (7.1 ^C) ^b	9/9 (8.1 ^A) ^A
5	CT/Mex/06 (1d) + CT/Mex/06 (3wks)	10/10 (8.7 ^B) ^a	10/10 (8.7 ^{A,B}) ^A	10/10 (10.7 ^B) ^a	10/10 (9.6 ^D) ^B	10/10 (10.4 ^D) ^b	10/10 (10.0 ^B) ^B

HI, hemagglutination inhibition; GMT, geometrical mean titer; rFPV-H7-AIV, rFPV-2155 vaccine; CT/Mex/06, A/Cinnamon Teal/Mexico/2817/2006 vaccine; d, day-of-age; wks, weeks-of-age.; *#positive/total (GMT includes only positive birds); [†]This group was not included in the statistical analysis.; Superscript letters outside the parenthesis indicate statistical comparisons among antigens tested within each experimental group.; Different superscript lowercase letters denote significant difference for HI titers between pre-challenge antigens tested within each group.; Different superscript uppercase letters denote significant difference for HI titers between post-challenge antigens tested within each group.; Superscript letters inside the parenthesis indicate statistical comparisons among experimental groups for each antigen tested.; Kruskal-Wallis test, $P < 0.05$; Mann-Whitney test, $P < 0.05$.

chickens (group 4). Interestingly, the bird from group 4 generally displayed more consistent staining and more severe microscopic lesions than the sham vaccinated birds (data not shown).

3.3. Reduction of virus shedding in the oropharynx and cloaca

Viral RNA titers were determined on OS and CS of 2 and 4 dpc by qRT-PCR (Table 1). Viral RNA was detected in 2 dpc-OS and -CS of all the sham vaccinated chickens (group 1) that died, with an overall mean titer of $10^{5.17}$ EID₅₀/ml (OS) and $10^{5.28}$ EID₅₀/ml (CS). At 4 dpc, none of the two surviving birds were shedding virus. Among the immunized groups, no or limited viral shedding was detected on 2 and 4 dpc. In the group vaccinated with CT/Mex/06 (1d) (group 4), at 2 dpc only 2 birds and 1 bird were positive for OS and CS, respectively, and at 4 dpc only 3 birds and 1 bird were positive for OS and CS, respectively, all of them with very low viral titers ($<10^{3.87}$ EID₅₀/ml). Similarly, only one bird in the rFPV-H7-AIV (1d) vaccinated group (group 2) shed virus both orally and cloacally at 4 dpc. Immunization with CT/Mex/06 at 3wks, receiving at 1d either rFPV-H7-AIV (group 3) or CT/Mex/06 (group 5), prevented challenge virus shedding. All vaccinated groups had statistically significant lower number of birds shedding virus and statistically significant lower titers than the sham vaccinated group.

3.4. Serology

Pre- (0 dpc) and post-challenge (14 dpc) sera were processed for HI assays to evaluate antibody levels against the vaccine seed antigens and the challenge antigen (Table 2). Sera from sham vaccinated chickens (group 1) were negative for all tested anti-AI antibodies on the day of challenge, and at 14 dpc the two surviving birds remained negative. In contrast, 80–100% of the birds of all the immunized groups elicited HI titers to the vaccine viruses and the challenge virus, both pre- and post-challenge, thus confirming the ability of sera from different vaccine combinations to cross-react among them. Interestingly, the bird from group 4 that died had low pre-challenge HI titers against the vaccine seed strain (\log_2 GMT = 4), and no titers against any of the other two antigens. Pre-challenge average antibody titers generally mirrored the vaccine combination implemented for each group. As expected, both pre- and post-challenge average antibody titers from 2-dose vaccinated groups were higher than their equivalent groups vaccinated with a 1-dose, being significantly different in all pre-challenge cases and in some post-challenge cases. Altogether, groups 3 (rFPV-H7-AIV [1d] + CT/Mex/06 [3wks]) and 5 (CT/Mex/06 [1d] + CT/Mex/06 [3wks]) were the vaccine combinations that induced higher average antibody responses for the three antigens tested, both pre- and post-challenge.

4. Discussion

Immunization of WL chickens with an rFPV-H7-AIV vaccine and an inactivated AIV vaccine, used in different combinations and ages, conferred protection against infection with the causative agent of the recent H7N3 HPAI epizootic in Mexico (A/Chicken/Jalisco/12283/12). The morbidity and mortality rates, rates of OS and CS shedding, and titers of challenge virus shed orally and cloacally were statistically different between any of the four vaccinated groups and the sham vaccinated group, but were not statistically different among the vaccinated groups.

Sham vaccinated birds exhibited high morbidity and mortality rates, as well as histopathological findings and extent of viral antigen indicative of HPAIV infection, according to the previously described high pathogenicity of the challenge virus [15]. On the contrary, all vaccine combinations conferred systemic immunity against AI as evidenced by significant protection from clinical signs and death. The prevention of clinical disease and death in immunized chickens challenged with HPAIV is well documented [13,16–20]. Such protection relies on a systemic humoral immune response that prevents hematogenous spread of the virus, replication in critical organs, and death of the individual [17]. However, an optimal vaccine strategy for AI also prevents virus replication in the respiratory and/or gastrointestinal tracts through local mucosal immunity, thus inhibiting viral shedding and bird-to-bird transmission [6,17]. In the current study, all immunization combinations significantly reduced not only the number of chickens shedding virus, but also the quantity of challenge virus shed from the oral and the cloacal routes. In particular, the groups receiving a single vaccine dose at 1d (groups 2 and 4) exhibited minimal or no viral shedding, whereas the groups receiving an additional vaccine boost at 3wks (groups 3 and 5) achieved total reduction of viral shedding levels and number of birds shedding virus, although these differences were not statistically significant. Such results presumably indicate that horizontal virus challenge transmission among birds would be totally prevented by any of the vaccination programs tested, even if a few chickens vaccinated with a single dose of inactivated vaccine (group 4) might still remain susceptible to AIV infection. Previously, single dose of inactivated vaccine at 7d meat turkeys provided limited protection against an Egyptian H5N1 HPAIV, suggesting that a second immunization at an older age would be recommended to confer protection in this species [21]. Similarly, protection against an Indonesian H5N1 HPAIV challenge was inadequate in ducks and geese after single dose of inactivated vaccine [22]. Likewise, in the current study, the presence of clinical signs in the single-inactivated-vaccine group (group 4) and the presence of viral shedding among some single vaccinated chickens (with either rFPV-H7-AIV [group 2] or inactivated vaccine

[group 4]) verses no clinical involvement or viral shedding in the twice-vaccinated chickens (groups 3 and 5), suggests that single vaccination in layers may not provide complete protection against Jalisco/12.

According to clinical protection and prevention of virus shedding, serological data also suggested that protection from HPAIV was similar but not equal among immunized groups. Characterization of the pre- and post-challenge HI titers identified antibody responses that were cross-reactive among the three tested seed strains, thus suggesting a broad range of immunity after AI vaccination with vaccine seed strains of diverse antigenic relatedness, as previously described [13,18,19,23]. Indeed, the A/Turkey/Virginia/66/02 isolate and the A/Cinnamon Teal/Mexico/2817/2006 isolate had 93.7% amino acid similarity in HA to each other, and 94.0% and 97.9% similarity with the challenge virus Jalisco/12, respectively, when compared by CLUSTAL W analysis. According to previous studies, HA similarities of at least 86% between vaccine seed strains and the challenge strain were needed to provide protection in chickens [23]. Besides, serum antibody responses appeared to correlate with protection against infection. In general, the birds that died had no or low pre-challenge HI titers ($\leq 4 \log_2$ GMT), although some vaccinated chickens that did not succumb to infection had similar low HI titers. Previously, moderate or high HI titers ($\geq 3 \log_2$ GMT) were associated with protection from mortality in H5-vaccinated young chickens when challenged with antigenically related H5 HPAIV, but this has not been confirmed a consistent positive predictor of survival [24,25]. Considering averages, booster vaccination (groups 3 and 5) significantly increased pre-challenge HI titers compared to their corresponding single-dose vaccination (groups 2 and 4, respectively). Overall, the present serological results indicate that vaccination with either the rFPV-H7-AIV or the inactivated CT/Mex/06 at 1d, followed by a boost of CT/Mex/06 at 3wks (groups 3 and 5), and conferred the best serological response to HPAIV.

The present study indicates that control of the recent Jalisco AIV infection could be achieved in different extent by using various combinations of an rFPV-H7-AIV vaccine and an inactivated CT/Mex/06 vaccine. Taking into account the several parameters assessed in the current study, and from a pragmatic point of view, a single dose of rFPV-H7-AIV vaccine administered at 1d could be the most effective immunization strategy against infection with A/Chicken/Jalisco/12283/12, but optimal protection may require a second dose of vaccine, administered to chickens in the field. The administration of the first vaccine dose may be a convenient option because of coinciding with routine hatchery procedures, which likely improves the biosecurity and quality control of vaccination process. In addition, the use of a heterologous vaccine with the N2 antigen would allow differentiating infected from vaccinated animals (DIVA) [25].

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